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Real-time PCR methods for the detection of blown pack spoilage causing *Clostridium* species; *C. estertheticum, C. gasigenes* and *C. ruminantium*

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Running heading: Real-time PCR methods for the detection of blown pack spoilage *Clostridium* species

1. Abstract

A set of real-time PCR methods for the detection of *C. estertheticum, C. gasigenes* and *C. ruminantium,* the causative agents of blown pack spoilage (BPS) in vacuum packaged beef, was developed. Robust validation of the sensitivity and specificity was carried out in the three matrices (beef meat drip, wet environmental swabs and dry environmental swabs) as encountered in our testing laboratory and against *Clostridium* strains (n=76) and non-*Clostridium* strains (n=36). It was possible to detect 4-5 spores per ml for *C. estertheticum,* 2 spores per ml for *C. gasigenes* and 8 spores per ml for *C. ruminantium,* without the need for enrichment of the samples. This high sensitivity is particularly important for the beef sector, not just for testing spoiled product but also in the early detection of contaminated beef and in validation of sporicidal cleaning procedures for critical pieces of equipment such as the vacuum packaging machine, which have the potential to contaminate large volumes of product.

2. Introduction

Although good hygiene and sufficiently low refrigeration temperatures (-1.5 °C) may be applied to control blown pack spoilage (BPS) of vacuum packaged beef primals (Milles et al., 2014), this is still is a major issue for the beef sector (Moschonas et al., 2009a). Beef carcasses are readily contaminated with the *C. estertheticum*, *C. gasigenes* and to a lesser extent *C. ruminantium* spores during dehiding and subsequent processing (Broda et al., 2009; Moschonas et al., 2009a). Vacuum packaging of deboned primals, often with a heat shrinkage step that promotes spore germination (Moschonas et al., 2011, Broda, 2007), and chilled (0 to 2 °C) storage creates the cold, anaerobic conditions in which these psychrophilic organisms may grow (Broda et al., 2003, Moschonas et al., 2010, Adams et al., 2012, Bolton et al., 2015). BPS, which usually occurs after 4-6 weeks of chilled storage, is characterised by the production of large volumes of gas (carbon dioxide) and offensive odour (hydrogen sulphide) and a metallic sheen. Meat spoiled in this way has no commercial value (Bolton et al., 2015).

Most BPS is caused by *C. estertheticum,* and *C. gasigenes* (Dainty et al., 1989, Broda et al., 2000, Moschonas et al., 2009a; Cavill *et al.,* 2011). However, *C. ruminantium* may also cause BPS in vacuum packed beef and lamb (Moschonas *et al*., 2009b, Moschonas *et al*., 2013). Control is currently reliant on good hygiene practices in the beef abattoir and boning hall, including routine decontamination of equipment with sporocidal agents such as peroxyacetic acid (2%, v/v). However, complete decontamination is often impossible as multiple surfaces in the beef plant may be heavily contaminated (Moschonas et al. 2009a). Moreover surfaces may be wet (the sporocidal treatment is often applied immediately after washing) and/or inaccessible (eg. the inside of the vacuum packaging machine). Despite this, there is no room

for error as one spore is sufficient to cause spoilage (Clemens et al., 2010, Moschonas et al., 2010). It is therefore important that beef processors are able to test and validate their disinfection procedures using methods capable of detecting very low levels of BPS spores.

Brightwell and Clemens (2012) recently published a direct real-time PCR method for *C. estertheticum* detection in meat, hide, blood, drip or environmental samples with a detection limit of 3 spores per ml. However, a cold temperature enrichment step was required prior to DNA extraction for faeces and soil samples and there was some cross-reactivity with other *Clostridium* spp. observed. There is currently no published real-time PCR method for either *C. gasigenes* or *C. ruminantium*. Indeed, a conventional PCR method to detect *C. ruminantium* is also unavailable. The objective of this research was therefore to develop and validate real-time PCR methods for the detection and quantification of *C. estertheticum*, *C. gasigenes* and *C. ruminantium* spores in meat drip and wet/dry swabs, the samples most often provided by the beef industry for testing.

3. Methods and Materials

3.1 Bacterial strains

Reference strains *Clostridium estertheticum* subsp. *estertheticum* (DSMZ 8809), *C. estertheticum* subsp. *laramiense* (DSMZ 14864) and *C. gasigenes* (DSMZ 12272) were purchased as freeze dried cultures from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunchweig, Germany). *C. ruminantium* (NCTC 13501) (National Collection of Type Cultures, Public Health England) was purchased from the NCTC. Each strain was revived under anaerobic conditions in 10 ml pre-reduced Peptone Yeast Extract Glucose Starch (PYGS) broth (Lund et al., 1990) and incubated for 3 weeks at

4°C. The purity of each strain was confirmed by plating out 0.1 ml aliquots on Columbia Blood Agar (CBA) (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (Cruinn Diagnostics, Dublin, Ireland) and incubated at 4°C for 3 weeks. Colony morphology was identified according to Moschonas *et al.*, (2009a); grey-white and opaque, circular, raised, convex, shiny, smooth and β-haemolytic for *C. gasigenes;* round with often coarsely granulated margins, smooth, slightly raised, cream-white to greyish and semi-transparent to opaque, nonhaemolytic for *C. estertheticum* subsp. *estertheticum* and β-haemolytic for *C. estertheticum* subsp. *laramiense. C. ruminantium* colony morphology was identified according to Moschonas (2009b). All strains were confirmed as strictly anaerobic Gram-positive rods. Purity was further checked by DNA extraction and 16S rRNA PCR detection (Broda et al., 2003a, Moschonas et al., 2009a).

Other bacterial strains used in this study were obtained from the culture collection at the Teagasc Food Research Centre, Ashtown, Dublin 15. *Clostridium* strains were maintained on Anaerobic Protect beads (Technical Service Consultants, UK). Non-*Clostridium* strains were maintained on Protect beads. All strains were stored at -80°C until required. All *Clostridium* strains were resuscitated in 30 ml PYGS broth. *C. estertheticum* strains were incubated at 10 °C for 3 weeks. *C. gasigenes* strains were incubated at 22 °C for 36 h and *C. ruminantium* strains were incubated at 25 °C for 5 days (Adam et al., 2013). All non-*Clostridium* strains were resuscitated in 30 ml Brain Heart Infusion Broth (Oxoid) and incubated at 30 °C and 37 °C, respectfully. One millilitre aliquots of each culture were centrifuged (5000xg, 10 min) and DNA was extracted as detailed in Section 3.3.

3.2 Spore preparation

Spore concentrates of *C. estertheticum* (DSMZ 8809), *C. estertheticum* subsp. *laramiense* (DSMZ 14864) , *C. gasigenes* (DSMZ 12272) and *C. ruminantium* (NCTC 13501) were

prepared by transferring 5 ml of exponentially growing culture to 100 ml of pre-reduced PYGS broth (Lund et al., 1990) and incubating at 4 °C for a minimum of 3 months to promote sporulation. Prior to inoculation all media was pre-reduced in an anaerobic cabinet for 24 h (Don Whitley Scientific Ltd, Shipley, UK) under an atmosphere of 100 % carbon dioxide at 20 °C. Spores were harvested using the method described by Moschonas *et al.* (Moschonas et al., 2010). Briefly, spore suspensions were recovered by centrifugation (7500xg, 4 °C, 10 min) and washed with saline (0.85 % NaCl) (Sigma Aldrich, Ireland). This was repeated a further three times. The washed spore suspension was then subsequently sonicated (40kHz for 15 min) in an ultrasonic waterbath (VWR International, USA) at room temperature and then subject to a further three sonication/ centrifugation/ wash cycles. The spores were then suspended in 10 ml saline and stored at -20 °C. Final spore numbers were estimated by preparing serial dilutions of the heat treated (80 °C, 10 min) spore suspensions in saline and plating out 0.1 ml aliquots on CBA supplemented with 5 % defibrinated horse blood, incubated anaerobically for 3 weeks at 4 °C.

3.3 DNA extraction from bacterial cultures and *Clostridium* spores

One millilitre of 10-fold *Clostridium* spore suspension or sample matrix spiked with spores was placed into a 1.5 ml eppendorf tubes, centrifuged (5000xg for 10 min) and resuspended in 180 µl lysozyme buffer and incubated for 30 minutes at 37 °C to breakdown the spore wall. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Ltd, Crawley, UK) according to the manufacturer's recommended protocol for DNA extraction (Bolton *et al.*, 2015).

3.4 Oligonucleotide primers and fluorescent-labelled probes

All primers and fluorescent labelled probes used for the standard and real-time PCRs were synthesized by Tib-molbiol (Berlin, Germany) and oligonucleotide sequences outlined in Table 2.

3.5 Selection of optimum primer and probe concentrations

The optimum concentrations for the primer and probes for each assay as detailed in Section 3.5 were determined by an initial screening of different concentrations (data not shown).

3.6 Real-time PCR conditions

All real-time PCR assays were performed using the Light Cycler 480 platform (LC 480) (Roche Diagnostics GmbH). The real-time PCR conditions were as follows for the three BPS organisms;

For the *C. gasigenes* assay, sequences of the 16S rRNA target were obtained from the NCBI database and were aligned using Clustal Omega software (EMBL-EBI, Cambridgeshire, UK). Following suggestions for Taqman primer/probe design (Tib-Molbiol, Berlin, Germany) the sequences in Table 2 were selected. Real-time PCR was performed in a 10µl reaction volume containing 0.5 µM primer and 0.2 µM probe, 2.8 µl H_2O, 5 µl Lightcycler 480 Probe Master mix (2X) (Roche Diagnostics) and 1µl of DNA to be tested. A positive and negative DNA control and no template control (NTC) were included in each real-time PCR run. The cycling protocol included a hot start of 95 °C for 10 minutes, followed by 45 cycles (95 °C for 10 s, 62 °C for 30 s and 72 °C for 1 s).

For the *C. ruminantium* assay; the 16S rRNA sequences were aligned using the SeqMan program in the LaserGene sequence analysis package (DNASTAR, Inc., Madison, WI).

Regions unique to *C. ruminantium* were identified, and oligonucleotide primers and probe were selected using the Primer3 program, by following the suggestions for TaqMan primer/ probe design (Applied Biosystems, Inc., Foster City, CA). After preliminary sensitivity and specificity testing with a number of candidate primer/probe sets, which included checking for potential cross-reactivity with the BLAST database, the UKCLOST set was selected (Table 2). Real-time PCR was performed in a 10µl reaction volume containing 0.2 µM primer and 0.1 µM probe, 2.8 µl H_2O, 5 µl Lightcycler 480 Probe master mix (2X) (Roche Diagnostics) and 1µl of DNA to be tested. A positive and negative DNA control and no template control (NTC) were included in each real-time PCR run. The cycling protocol included a hot start of 95 °C for 10 minutes, followed by 45 cycles (95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s).

For the *C. estertheticum* assay, the primers and probe (Table 2) were obtained from the real-time PCR method developed by Brightwell and Clemens (2012) and have been optimised for use on the LC480. Real-time PCR was performed in a 10 µl reaction volume containing 0.3 µM primer and 0.1 µM probe, 2.8 µl H_2O, 5 µl Lightcycler 480 Probe master mix (2X) (Roche Diagnostics) and 1 µl of DNA to be tested. A positive and negative DNA control and no template control (NTC) were included in each real-time PCR run. The cycling protocol included a hot start of 95°C for 10 minutes, followed by 45 cycles (95 °C for 10 s, 65 °C for 30 s and 72 °C for 1 s).

3.7 Real-time PCR specificity

All three assays were tested against a panel of purified DNA from non-*Clostridium* strains (n=36) (Table 1) and a panel of *Clostridium* strains (n= 76; 15 *C. estertheticum*, 31 *C. gasigenes* and 29 *C. ruminantium*) to demonstrate the specificity of the assays for cross-reactivity.

3.7 Real-time PCR sensitivity and efficiency

Three sets of ten-fold serial dilutions were performed using DNA extracted from *C. estertheticum, C. gasigenes* and *C.ruminantium* reference strains. The DNA was diluted using AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0) from the Qiagen DNasy Blood and tissue kit. This serially diluted DNA was used to create the standard curves. The DNA concentration was measured using a Nanodrop^{TM} 1000 (Labtech International, Ringmer, UK). The number of *Clostridium* genomic equivalents (GE) were calculated as follows: $m=n$ x (1.013×10^{-21} g bp^{-1}) where *m* is the mass and *n* is the number of base pairs. The number of Mbp for *C. estertheticum, C. gasigenes and C. ruminantium* was determined for the three reference strains by next generation sequencing (Illumina) to be 4787 Mbp, 4113 Mbp and 3518 Mbp, respectively (data unpublished). According to the equation, the average weight of one *Clostridium* genome is 4 fg. The number of (GE μl^{-1}) were calculated and adjusted in AE buffer, by diluting the DNA to set up a range of standards from 10 to 10^6 GE per µl, which were run in triplicate.

3.8 Validation *of C. estertheticum, C. gasigenes* and *C. ruminantium* real-time PCR methods

Serial spore dilutions of each BPS *Clostridium* species was used to spike meat drip and environmental swabs (wet and dry swabs). All matrices were tested for absence of *C. estertheticum, C. gasigenes* and *C. ruminantium* before artificial inoculation by PCR. A triplicate set of 1 ml of meat drip and 30 ml universals containing the swab samples were placed in both 9 ml MRD (Fannin, Oxoid) and 9 ml pre reduced Peptone Yeast Glucose Starch Broth (PYGS) in an anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, UK). A one millilitre aliquot of each 10 fold serial spore suspension was then added to each tube. DNA was extracted as detailed in Section 3.3.

4. Results

4.1 Real-time PCR specificity

The specificity of each assay was tested against a panel of non–*Clostridium* DNA (Table 1), as well as against a *Clostridium* specific DNA panel. For the *C. estertheticum* assay, with a Tm of 65 °C for 30 s the discriminatory power of the assay was increased, allowing specific detection of *C. estertheticum* strains. A Tm of 62 °C for 30 s and 60°C for 50 s allowed specificity for *C. gasigenes* and *C. ruminantium* strains, respectively. For the non-Clostridial panel no non-specific amplification was observed in any of the three assays below 35 cycles.

4.2 Real-time PCR sensitivity

The limit of detection and efficiency were determined for each of the three assays and is shown in Table 3. All of the concentrations tested produced standard curves that had an efficiency ranging from 2.007-2.184. The dynamic range extended from ~ 10^6 to ~10^1 GE per reaction for each of the strains. The limit of detection was calculated was calculated to be 1-17 GE for *C. gasigenes* DSMZ 12272, 1-12 GE for *C. ruminantium* NCTC 13501, < 10 GE for *C. estertheticum* DSMZ 8809 and 1-48 GE for *C. estertheticum* DSMZ 14864. From the standard curves generated the error was calculated using the Lightcycler 480 Relative Quantification Software and was 0.05 or lower for each species.

4.3 Detection of BPS *Clostridium* spp. in a complex matrix using real-time PCR

Table 4 shows the results for the spiking of each BPS *Clostridium* spp. into beef meat drip and environmental swabs (wet and dry), samples that are typically used to test meat processing plants and/or product for the presence of BPS *Clostridium* species. All matrices were analysed for the presence of BPS *Clostridium* species by PCR and tested negative prior to inoculation.

For each real-time PCR assay, target DNA was amplified for all 10-fold spore dilutions (10^6-10^0) without a three week enrichment in both MRD and PYGS media. The limit of detection was approximately five spores per ml for *C. estertheticum* DZMZ 8809, four spores per ml for *C. estertheticum* DSMZ 14864, two spores ml^{-1} for *C. gasigenes* DSMZ 12272 and eight spores per ml for *C. ruminantium* NCTC 13501. There was no amplification of the no template control in any assay.

5. Discussion

Our study developed and validated real-time PCR methods to test meat drip and environmental swabs for the presence of *C. estertheticum, C. gasigenes* and *C. ruminantium*. The detection limits were 4-5 spores per ml for *C. estertheticum,* 2 spores per ml for *C. gasigenes* and 8 spores per ml for *C. ruminantium,* without the need for enrichment of the samples. A similar real-time PCR assay was previously developed by Brightwell and Clemens, (2012) but only for the detection of *C. estertheticum* and had a detection limit of 3 spores per ml for meat, hide, blood/drip and environmental samples. Thus our study provides the first published real-time PCR assay for the detection of *C. gasigenes* or *C. ruminantium*.

Prior to these developments, the majority of methods available to detect BPS *Clostridium* spp. were based on conventional PCR and are therefore not quantitative. Broda *et al*. (2003), for example, developed detection methodologies for *Clostridium estertheticum* subsp. *estertheticum, C. estertheticum* subsp. *laramiense* and *C. gasigenes* on minced beef, with a limit of detection of 10^4 CFU/g. Other molecular methods, such as the PCR-RFLP method developed by Broda *et al.,* (2000) are based on universal eubacterial primers and may therefore only be used for confirmatory purposes.

These new real-time PCR methods are particularly important for the beef sector, for the early detection of contaminated beef and in the validation of sporicidal cleaning procedures for critical pieces of equipment such as the vacuum packaging machine, which have the potential to contaminate large volumes of product (Moschonas et al., 2009a)

6. Conclusion

This study developed real-time PCR methods for the detection of low concentrations of BPS-causing *C. estertheticum C. gasigenes* and *C. ruminantium* spores in meat drip and environmental swabs, without the need for an enrichment step. The low detection limits (approximately five spores ml^{-1} for *C. estertheticum* DZMZ 8809, four spores ml^{-1} for *C. estertheticum* DSMZ 14864, two spores ml^{-1} for *C. gasigenes* DSMZ 12272 and eight spores ml^{-1} for *C. ruminantium* NCTC 13501) mean these assays will find application in the early detection of BPS in beef primals and in the validation of disinfection of critical pieces of equipment, such as the vacuum packaging machine. Future work should investigate the development of a multiplex assay to detect all commercially relevant BPS *Clostridium* spp.

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Conflict of interest

No conflict of interest declared.

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Table 1. Thirty-six non-Clostridial species associated with meat processing.

Bacterial Species	**Source**
Acinetobacter calcoaceticus	ATCC 23055[a]
Aeromonas hydrophila	ATCC 35654
Arthobacter globiformis	ATCC 8010
Bacillus cereus	NCTC 07464[b]
Citrobacter diversus	CCFRA 7119[c]
Citrobacter freundi	NCTC 09750
Citrobacter freundi	NCTC 8090
Citrobacter koseri	NCTC 10768
Cronobacter sakazaki	NCTC 11467
Enterobacter aerogenes	NCTC 10006
Enterobacter agglomerans	NCTC 09381
Enterobacter cloacae	NCTC 11937
Enterobacter intermedius	NDC 427
Enterococcus faecalis	NCTC 12697
Enterococcus faecium	ATCC 35667
Escherichia coli	ATCC 25922
Escherichia coli	NCTC 09001
Escherichia coli	NDC 544[d]
Klebsiella oxytoca	ATCC 43086
Klebsiella aerogenes	ATCC 13.883

Leuconostoc mesenteroides	ATCC 8293
Proteus mirabilis	DSM 4479[e]
Pseudomonas aeroginosa	NCTC 12903
Pseudomonas aeruginosa	NCTC 12469
Pseudomonas aeruginosa	P20[f]
Pseudomonas fluorescens	P30
Pseudomonas fluorescens	P40
Pseudomonas putida	P50
Pseudomonas putida	P60
Pseudomonas putida	P70
Pseudomonas fragi	DSM 3456
Pseudomonas putida	ATCC 49128
Staphylococcus epidermis	AFRC
Staphylococcus haemolyticus	ATCC 29970
Staphylococcus saprophyticus	ATCC 15305
Streptococcus lactis	NCDO 2003[g]

[a] ATCC® American Type Culture Collection.

[b] NCTC National Collection of Type Cultures.

[c] CCFRA Campden and Chorleywood Food Research Association.

[d] NDC National Diagnostic Centre, NUI Galway, Ireland.

[e] DSMZ German Collection of Microorganisms and Cell Cultures

[f] AFRC Ashtown Food Research Centre, Teagasc, Dublin 15, Ireland.

[g] NCDO National Collection of Dairy Organisms c/o NCIMB Ltd., Aberdeen, Scotland, United Kingdom.

Table 2. Primers and probes used for the real-time PCR methods.

BPS Clostridium Species	**Primer name**	**Primer Sequence**	**Source**
C. estertheticum	TMF (forward primer)	5’ CGG CGG ACG GGT GAG TAA C 3’	Brightwell and Clemens, 2012
	TMR (reverse primer)	5’ CGG GTC CAT CTC AAA GTG RAA CT 3’	Brightwell and Clemens, 2012
	Probe	5’ FAM- CGT GGG TAA CCT GCC TCA AAG AGG GG-BBQ 3’	Brightwell and Clemens, 2012
C. gasigenes	16SDB_for (forward primer)	5’ GAG AGG AGT TCT TCG GAA CGA 3’	Designed in-house
	16SDB-A (reverse primer)	5’ GGA TTT CTC CTT TAA TTG CTG CT 3’	Designed in-house
	16sDB_TM Probe	5’ FAM-ATG CGA AAC TGC AAT GTT ATG CGGT—BBQ 3’	Designed in-house
C. ruminantium	UKClost1-fw	5’TGA CGG TAC TTG AGG AGG AAG3’	Designed in-house

	UKClost1-rev	5’CGC CTT CGC AAC TGG TAT TC3’	Designed in-house
	UKClost1	5’FAM- CGG CTC AAC CGT AGT AAG CCT TTG AA- BBQ3’	Designed in-house

Table 3. Dynamic range and sensitivity of the three developed real-time PCR assays for BPS *Clostridium* species. The PCR efficiency and error were calculated for each of the standard curves generated from serial dilutions of DNA extracted from the reference strains of *Clostridium.*

Clostridium strain	*C. estertheticum* subspp. *estertheticum* (DSMZ 8809)		*C. estertheticum* subspp. *laramanise* (DSMZ 14864)		*C. gasigenes* (DSMZ 12272)		*C. ruminantium* (NCTC 13501)	
GE/PCR[a]	1.06E+06(TWS)[b]		4.80E+06(TWS)		1.78E+06(TWS)		1.21E+06(TWS)	
	Cp[c]	SD[d]	Cp	SD	Cp	SD	Cp	SD
10^6	16.2	0.06	15.94	0.11	16.62	0.16	9.15	0.18
10^5	19.34	0.14	18.81	0.15	19.72	0.36	12.4	0.17
10^4	22.78	0.11	22.06	0.08	22.83	0.19	15.3	0.21
10^3	26.17	0.11	25.48	0.24	26.32	0.62	18.57	0.28
10^2	29.67	0.02	29.10	0.42	28.7	0.11	21.4	0.07
10^1	31.08	0.16	33.06	0.04	31.62	0.21	26.62	0.16
10^0	34.61	0.02	35.17	0.01	34.4	0.33	29.16	0.11
PCR	2.007		2.184		2.025		2.086	

Efficiency[e]				
Error[f]	0.00785	0.0275	0.05	0.0212
Slope	-3.306	-3.224	-3.263	-3.113

[a]GE: genome equivalent: the weight of one *Clostridium* genome is approximately 4 fg, the GE is calculated by dividing one *Clostridium* genome by the initial concentration of DNA (ng/µl).

[b]The top working standard (TWS) is the initial GE concentration of DNA (ng/µl) before serial dilutions.

[c]Cp: Crossing point is proportional to the initial concentration of the template, the mean of 3 Cps.

[d]SD: standard deviation

[e]PCR efficiency (calculated by $E = (10^{-1/slope})-1$.

[f]Error allowable value ≤ 0.1.

Table 4. Real-time PCR detection in meat drip and environmental swabs (wet and dry).

		Sample Matrix					
***C. estertheticum* subspp. *estertheticum* (DSMZ 8809)**		**Meat Drip**		**Wet swab**		**Dry swab**	
		MRD	**PYGS**	**MRD**	**PYGS**	**MRD**	**PYGS**
Log number of spores/ml	**Spore concentration**	Cp	Cp	Cp	Cp	Cp	Cp
6.70	5.0×10^6	15.69	15.47	16.26	16.34	16.15	16.19
5.70	5.0×10^5	17.02	17.17	19.35	19.19	19.35	19.19
4.70	5.0×10^4	20.23	19.96	22.88	22.80	22.88	22.80
3.70	5.0×10^3	23.27	23.36	26.27	26.19	26.27	26.19
2.70	5.0×10^2	26.87	26.32	29.64	29.67	29.64	29.67
1.70	5.0×10^1	30.32	30.13	31.15	31.26	31.94	31.86
0.70	5.0×10^0	33.45	33.56	34.85	34.17	34.07	34.14
Positive DNA Control		16.62					
Blank		ND					
***C. estertheticum* subspp. *laramenise* (DSMZ 14864)**		MRD	PYGS	MRD	PYGS	MRD	PYGS
		Cp	Cp	Cp	Cp	Cp	Cp
6.59	3.8×10^6	15.86	15.90	14.46	14.54	14.65	14.74
5.59	3.8×10^5	18.66	18.82	17.10	17.17	17.10	17.17
4.59	3.8×10^4	22.13	21.98	20.33	20.58	20.33	20.58
3.59	3.8×10^3	25.63	25.61	23.60	23.93	23.60	23.93
2.59	3.8×10^2	28.89	28.83	27.18	27.28	27.18	27.28

1.59	3.8 x 10^1	30.72	31.13	30.01	29.98	30.11	30.07
0.59	3.8 x 10^0	34.61	34.10	33.25	33.14	33.22	33.41
Positive DNA Control		13.69					
Blank		ND					
***C. gasigenes* (DSMZ 12272)**		MRD	PYGS	MRD	PYGS	MRD	PYGS
		Cp	Cp	Cp	Cp	Cp	Cp
6.23	1.6 x 10^6	16.81	16.89	16.81	16.48	16.57	16.80
5.23	1.6 x 10^5	19.57	19.60	19.99	19.51	19.52	20.13
4.23	1.6 x 10^4	22.12	22.29	22.91	22.70	22.97	22.84
3.23	1.6 x 10^3	24.74	25.17	25.64	25.88	25.94	26.76
2.23	1.6 x 10^2	27.04	27.14	27.51	28.70	28.95	29.10
1.23	1.6 x 10^1	30.15	30.24	30.44	31.86	31.59	31.72
0.23	1.6 x 10^0	33.05	33.20	33.17	34.50	34.59	34.18
Positive DNA Control		17.15					
Blank		ND					
***C. ruminantium* (NCTC 13501)**		MRD	PYGS	MRD	PYGS	MRD	PYGS
		Cp	Cp	Cp	Cp	Cp	Cp
6.83	6.7 x 10^6	9.09	9.04	9.13	8.87	8.86	8.91
5.83	6.7 x 10^5	12.23	12.19	12.18	12.32	12.50	12.47
4.83	6.7 x 10^4	15.20	15.07	15.09	15.74	15.86	15.92
3.83	6.7 x 10^3	18.21	17.94	17.76	18.93	18.68	18.72
2.83	6.7 x 10^2	20.81	20.81	20.97	20.98	21.96	21.47
1.83	6.7 x 10^1	24.10	24.10	24.06	23.95	24.10	24.05

0.83	6.7×10^0	27.51	27.46	27.31	26.84	27.02	27.07
Positive DNA Control		8.87					
Blank		ND					